Although 2',3'-cyclic nucleotide 3'-phosphohydrolase (EC 3.1.4.37-CNPase) activity was initially described in bovine spleen and pancreas, 30 years have now elapsed since it was realized that high levels of activity were present in the vertebrate nervous system [1]. The enzyme hydrolyses 2',3'-cyclic nucleotides (but not 3',5'-cyclic nucleotides) to produce exclusively the 2'-derivatives. This property, together with the lack of requirement of a metal ion cofactor and the ability to hydrolyse all four 2',3'-cyclic nucleotides, distinguishes CNPase from most other known phosphodiesterases [2, 3]. CNPase also hydrolyses oligonucleotides with a 2',3'-cyclic terminus [2, 3]. Purine-containing nucleotides are hydrolysed at a faster rate than pyrimidine-containing nucleotides [3]. Fluorescent derivatives of 2',3'-cyclic nucleotides or 2',3'-cyclic NADP are commonly used as in vitro substrates of the enzyme; $K_m$ values for such substrates (depending on the substrate and the source of the CNPase) range from 0.2 mM to as high as 10 mM [3]. What appears to be true CNPase activity can be detected in a wide range of vertebrate organs; however, the specific activity in brain and spinal cord is usually at least one and often two orders of magnitude greater than that found in other tissues [3]. During evolution, significant CNPase activity in the central nervous system (CNS) first appears in amphibians.
The highest known specific activities of CNPase occur in Xenopus laevis spinal cord [3]. An enzyme apparently identical to CNPase occurs in wheat germ [3] and, recently, an interesting report has shown CNPase activity in an RNA virus [4]. The evidence localizing CNPase to CNS myelin is impressive. This evidence is based on subcellular fractionation, developmental studies, neurological mutants, and on histochemical and immunohistochemical studies summarized elsewhere [2, 3]. Furthermore, CNPase appears to be associated with loosely compacted 'heavy' myelin rather than 'mature' multilamellar 'light' myelin. The evidence for this comes firstly from centrifugation of myelin fractions in sucrose gradients, which has shown CNPase activity in an RNA virus [4]. A further localization of CNPase is in the membranes of photoreceptor cells in the retina [7]. Surprisingly, this intriguing finding seems to have been largely ignored since its original publication.

Examination of CNS myelin from several species using SDS-PAGE shows CNPase running as two polypeptides of about 46 kDa and 48 kDa, which have been named CNP1 and CNP2, respectively [3]. The ratio of CNP1 to CNP2 varies between species; for example, in the mouse and rat the ratio is about 10:1, in rabbit CNS myelin about 1:1 [5], and in bovine myelin about 1:2 [3]. These two polypeptides represent the protein doublet previously described as Wolfram protein [5] and both can be shown to exhibit CNPase enzyme activity after transfer to nitrocellulose filters [8], and to react with antisera raised against pure CNPase [3]. Bovine CNPase, when chromatographed under non-denaturing conditions, shows an apparent molecular weight of 90–96 kDa, suggesting that native CNPase exists as a dimer [3]. If the variation in apparent relative proportions of CNP1 and CNP2 between different species and the estimated native molecular weight are both correct, then this suggests that within CNS myelin CNPase may exist as a mixture of homodimers and heterodimers of CNP1 and CNP2 or even possibly in a monomeric form.

CNPase has been purified to homogeneity from bovine, rat and human brain and exhibits the characteristic CNP1 and CNP2 doublet on SDS-PAGE in each case [3]. Partial amino acid sequences obtained from peptide digests of bovine and rat enzymes (in each case a mixture of CNP1 and CNP2) confirmed that the total primary amino acid structures deduced from cDNA sequences [9–11]. The cDNAs coding for bovine CNPase were sequenced independently by two groups [9, 10] while a third group sequenced a cDNA coding for rat CNPase [11]. More recently the sequences of cDNAs for human [12] and mouse CNPase have been reported [13, 14]. Fig. 1 shows the DNA sequence and deduced amino acid sequence of human CNPase in relation to the intron–exon arrangements of the human CNPase structural gene [15].

Several features of the primary amino acid sequence of CNPase are of interest. Firstly CNPase does not show typical hydrophobic transmembrane domains (Fig. 1) and is therefore considered to be attached to the inner cytoplasmic surface of the oligodendrocyte membrane rather than spanning it. Secondly, CNPase shows a CAAX motif at the C-terminus, which is highly conserved in all four species so far sequenced (i.e. bovine, rat, mouse and human) as CT11 in each case. The CAAX motif is found at the C-terminus of p21ras, some ras-associated proteins, the γ-subunit of some heterotrimeric G-proteins and nuclear lamins [17]. The cysteine residue is covalently linked to a prenyl derivative, which is followed by removal of the three AAX amino acids and methyl esterification of the now C-terminal modified cysteine. The p21ras proteins are modified with a C15 farnesyl residue, and other CAAX proteins containing leucine as the C-terminal amino acid are modified by a C20 geranylgeranyl residue [17]. This C-terminal processing of ras and ras-related proteins — along with a second signal — is necessary for efficient membrane association of CAAX proteins. This second signal is either a polybasic domain (six consecutive lysine residues in p21Kras) or palmitoylation of a cysteine residue elsewhere in the CAAX protein. Incubating rat C6 glioma cells with radioactive mevalonolactone (a precursor for isoprenoid synthesis) resulted in labelling of CNP1 on subsequent electrophoresis and autoradiography [18]. However, the covalent attachment site and the nature of the isoprenoid modification was not determined [18], and possible labelling of CNP2 as well as CNP1 could not be excluded [18]. CNPase does not contain a polybasic domain (Fig. 1); however,
Fig. 1

The DNA and amino acid sequence of human CNPase in relation to the intron-exon arrangement of the human structural gene

The gene consists of four exons (0, 1, 2, 3), the first exon (exon 0) coding for an initiator methionine only. Use of this exon produces a protein with a 20 amino acid N-terminal extension compared to initiating translation at the ATG codon boxed at nucleotides 61–63. The cyclic-AMP-dependent protein kinase consensus sites conserved in all known CNPase sequences are boxed. The two GXGXXGK motifs seen in GTP-binding proteins and the C-terminal CAAX motif are underlined. * shows an A→G transition from the nucleotide sequence of an independent human cDNA clone [12]. See [15] for further details.

The DNA and amino acid sequence of human CNPase in relation to the intron-exon arrangement of the structural gene. The gene consists of four exons (0, 1, 2, 3), the first exon (exon 0) coding for an initiator methionine only. Use of this exon produces a protein with a 20 amino acid N-terminal extension compared to initiating translation at the ATG codon boxed at nucleotides 61–63. The cyclic-AMP-dependent protein kinase consensus sites conserved in all known CNPase sequences are boxed. The two GXGXXGK motifs seen in GTP-binding proteins and the C-terminal CAAX motif are underlined. * shows an A→G transition from the nucleotide sequence of an independent human cDNA clone [12]. See [15] for further details. The enzyme is known to be palmitoylated at an undefined site [19]. Other features of the primary sequence of human CNPase [3] are a GXGXXGK motif at amino acids 57–63 (numbering from the second initiator methionine; see later) and again at amino acids 365–361. This motif is found at phosphoryl-binding sites in GTP-binding proteins [16]. The first of these sequences (GLPGSGK) is conserved in all four known CNPase sequences, and the second (GELFSGK) is conserved in bovine CNPase but shows an S-P substitution in rat and mouse CNPase [9, 11, 14]. Of the three potential cyclic-AMP-dependent protein kinase consensus sites originally reported in the bovine CNPase sequence [9, 10], only the KKSS sequence at 175–179 is conserved between all four known CNPase sequences (Fig. 1).

All purified CNPase proteins so far examined have blocked N-termini and in the initial cloning of all four species of CNPase [9–12] the N-terminal sequence was assumed to be MSSSSGA (see Fig. 1). Additionally only a single CNPase mRNA was apparent in bovine and human brain [9, 10, 12], whereas at least two closely spaced mRNAs were detected in rat and mouse brain [11, 14]. The sequence of bovine retinal CNPase was found to be identical to the brain form [19a]. The origin of the CNP1 and CNP2 polypeptides seen on SDS-PAGE could potentially have been from two separate CNPase genes, and the presence of one gene on chromosome 11 and one gene on chromosome 3 was suggested in the mouse [20]. We have examined human genomic DNA using a panel of somatic cell hybrids and fluorescence in situ hybridisation and have localized the human CNPase structural gene to chromosome 17q21, this being homologous to chromosome 11 in the mouse [15]. No evidence was found for a second human CNPase locus [15]. The discovery of a cDNA in a mouse brain library encoding a CNPase with an N-terminal extension of 20 amino acids [14] enabled the identification of a 5′-exon (exon 0) in the mouse CNPase structural gene which encoded a single initiator methionine. The use of this upstream start site and an alternative splice site in exon 1 would allow the production of a larger polypeptide (CNP2) from a smaller mRNA [14]. The N-terminal extension contains a high proportion of basic amino acids, and CNP2 (at least in rabbit CNS myelin) is known to be considerably more basic than CNP1 [5]. We have analysed the human structural gene for CNPase and find a similar structure to the mouse gene (Fig. 1). The human gene is slightly larger than the mouse because of the Alu sequence repeats in intron 1 (not shown). Although the structure of the human gene would allow for the production of CNP1 and the larger CNP2 by an alternative splicing mechanism, there is (unlike the
Okadaic acid is a potent inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A), two of the four serine/threonine protein phosphatases in the cytoplasm of eukaryotic cells [25]. Inhibition of PP2A occurs at 1 mM okadaic acid, and of PP1 at 1 μM [25]. The minimum concentration of okadaic acid to produce stimulation of MBP and CNPase phosphorylation (which is assumed to be caused by inhibition of phosphatases concomitantly removing phosphate groups) is about 5 μM. Further investigation of the phosphatases present in CNS myelin and their sensitivity to okadaic acid is required. Other agents that increase MBP phosphorylation are chloroform (reported to activate protein kinase C; see [22]) and the non-hydrolysable GTP analogue, GTP-γ-S. The latter, at concentrations of 0.2 mM, produces a marked stimulation of the in vitro phosphorylation of both MBP and CNPase, but only in the presence of okadaic acid. The stimulation observed with GTP-γ-S is not seen with equivalent concentrations of UTP or CTP.

Stimulation of myelin protein phosphorylation by a non-hydrolysable GTP analogue suggests the presence of signal-transducing GTP-binding proteins [16]. These belong to two major families, the heterotrimeric G-proteins and the low-molecular-weight (20–25 kDa) GTP-binding proteins which form various branches of the ras superfamily [16]. Heterotrimeric G-proteins consist of GTP-binding α subunits complexed with β and γ subunits. Activation of an appropriate receptor leads to displacement of GDP bound to the α subunit by GTP, leading to dissociation of the GTP–α-subunit complex from the β–γ subunits. The GTP–α-subunit complex interacts with an effector molecule (e.g. an ion channel, adenyl cyclase, phospholipases etc.) to amplify the incoming receptor signal. Hydrolysis of bound GTP to GDP by the G-protein α subunit dissociates the complex from the effector and terminates the amplification signal [16]. The GDP–α–subunit complex reassociates with the β–γ subunit complex to complete the cycle [16]. The rate of hydrolysis of GTP to GDP (and hence the extent of signal amplification) is determined by the individual type of Gα subunit, for which at least nine separate structural genes are known to exist [16]. Signal transduction by the monomeric ras superfamily of proteins is essentially similar (i.e. activation by exchange of GDP for GTP and inactivation by GTP hydrolysis), except that the rate of GDP–GTP exchange and of GTP hydrolysis is determined by ancillary exchange-promoting and GTPase activating proteins [16]. Gα subunits are ADP-ribosylated at a specific arginine residue by cholera
toxin, Gi and Go \( \alpha \) subunits by pertussis toxin, and \( \alpha \) subunits of retinal transducins by both toxins. Some members of the ras superfamily (rho proteins) are ADP-ribosylated by botulinum C3 toxin [16].

The initial evidence for heterotrimeric G-proteins in CNS myelin was produced several years ago [26]. Myelin, which was apparently devoid of adenyl cyclase activity, was unexpectedly found to be a source of a component that could restore guanine nucleotide sensitivity when combined with the catalytic subunit of the enzyme [26]. Several proteins in rat CNS myelin could also be shown to be ADP-ribosylated by cholera toxin [26]. A recent study [27] has produced immunological evidence for the presence of Go \( \alpha \), Gi \( \alpha \), Gs \( \alpha \) and ras proteins in mouse and rat myelin and, using the nitrocellulose blot method [28], has demonstrated the presence of four low-molecular-weight GTP-binding proteins. All isoforms of rat and mouse MHP were ADP-ribosylated by cholera toxin but not by pertussis toxin [27]. A second report using bovine CNS myelin [29] has also demonstrated the presence of Go \( \alpha \), Gi \( \alpha \) and Gs \( \alpha \), as well as of three low-molecular-weight GTP-binding proteins. A 43 kDa cholera substrate and two 40 kDa pertussis toxin substrates were detected [29]. The presence of these signal-transducing GTP-binding proteins was suggested to be related to muscarinic receptors proposed to be present in CNS myelin [29].

We have been examining human and rabbit CNS myelin and other brain subcellular fractions for ADP-ribosylating substrates and low-molecular-weight GTP-binding proteins. In our hands, cholera toxin ADP-ribosylates two substrates in CNS myelin. The first is of about 44 kDa and clearly migrates more rapidly than the smaller CNPase isoform (CNP1) and the second co-electrophoreses with MBP. These substrates appear to be confined to CNS myelin. Pertussis toxin ADP-ribosylates a substrate of approximately 40 kDa which appears to be present in all fractions; additionally, there are fainter low-molecular-weight substrates that are larger than MBP. There are at least three low-
molecular-weight GTP-binding proteins which (if run on 15% polyacrylamide gels) are clearly of higher molecular weight than MBP (18.5 kDa). When myelin is examined by sucrose density gradient centrifugation, these low-molecular-weight GTP-binding proteins are most prominent in the heavier more rapidly sedimenting fractions i.e. those fractions that show the highest specific activity of CNPase. The presence of these specific kinases and GTP-binding proteins in CNS myelin indicates the existence of at least two signal-transducing mechanisms responding to as yet unidentified primary effectors.

Note added in proof
A recent report [30] has shown that an antisera raised against a synthetic peptide representing the predicted N-terminal extension of CNP2 exclusively stains the larger isoform on Western blots of mouse, rat, human and bovine myelin, providing further evidence for the alternative splicing mechanism discussed above.

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